Remarks/Arguments

Applicants wish to thank Examiner Oh and supervisory Primary Examiner Hartley for the courtesy of the interview extended the undersigned attorney during which the outstanding rejections over the prior art rejections were discussed.

New claims 36 through 47 have been added to the application and are fully supported by the application as originally filed and as would be understood by one of ordinary skill in the art to which the invention pertains. Applicants most respectfully submit that all of the claims in the application are in full compliance with 35 USC 112 and are clearly patentable over the references of record.

The Examiner has rejected claims 14-32 and 33-35 under 35 USC 103(a) as being unpatentable over the combination of Green et al. in view of Paul. These rejections have been carefully considered but are most respectfully traversed in view of the amendments to the claims.

Applicants appreciate that new Examiners are now in charge of this application and wish to reiterate that it is the core of the present invention that the carbohydrates of the component A are structurally different from the carbohydrates of the component B. In the case of example 1 of the present application, the carbohydrates of the component A are galacto-oligosaccharides whereas the carbohydrates of the component B are fructo-oligosaccharides which are clearly structurally different from each other as would be appreciated by one of ordinary skill in the art to which the invention pertains.

Applicants most respectfully submit that the GREEN reference (US 5 792 754) teaches nowhere a combination of structurally different carbohydrates as this would be appreciated by one of ordinary skill in the art to which the intention pertains. Applicants believe that the Examiner misinterprets the teaching of

example 1 of the GREEN reference. There it is disclosed that raftilose (hydrolyzed inulin) is used. When hydrolyzing inulin you obtain FOS of different chain lengths. In other words the example 1 contains short chain FOS and long chain FOS. These FOS have (despite the chain length) the same structure and can therefore not be considered as belonging to component A and component B at the same time since the component A has to be structurally different from the component B (see above).

It is not claimed by the present invention to administer carbohydrates of different chain lengths only such as for example FOS having a chain length or a DP of 1 to 6 and a DP of 7 to 100.

According to the present invention the carbohydrates of component A have a DP of 1 to 7 whereas the carbohydrates of component B have a DP of 7 to 100. In addition carbohydrates belonging to component A and carbohydrates belonging to component B have to be structurally different. This very important, additional requirement is not met by example 1 of the GREEN reference. GREEN describes indeed hydrolyzed inulin with a DP of 1 to 6 and a DP of 7 to 100. Such an inulin, however, does not meet the claim limitations of the present application since the structure of inulin of DP 1 to 6 is the same as that one of inulin of DP 7 to 100. In this regard, Applicants wish to note that applicants cannot perform comparative tests using inulin (comprising FOS with a DP of 1 to 7 and FOS with a DP of 8 to 100) as a comparative test system due to ethical reasons since the corresponding studies have to be performed with preterm infants. However, the tests described in the article "Supplementation of a bovine milk ..." have been performed with a more or less similar placebo, i.e. maltodextrin which also contains long chain and short chain carbohydrates of the same structure.

In this context applicants wish to direct the Examiner's attention example 1 of the present application which employs indeed inulin. However, the low

molecular oligosaccharides and therefore inulin of a DP of 1 to 6 has been removed by physical separation. Even if inulin of a DP of 1 to 6 would be present this does not effect the core of the present invention since inulin (=FOS) is present together with GOS. Galacto-oligosaccharides are not obtained when inulin is hydrolyzed. Consequently the composition of example 1 of the present invention is different from the teaching of the GREEN reference.

To say it once more the comparataive test data do not show a combination of GOS and inulin (i.e. short chain FOS and long chain FOS). The comparative data rather show the combination of GOS and long chain FOS only.

While Applicants have retained the broader aspects of the claims previously presented, as these are believed to be patentable over the prior art, note also the more limited claims, such as claim 44 where it is stated that at least 60 wt.-% and in particular 80 to 100 wt.-% of the carbohydrates of the carbohydrate component A belong to the galacto-oligosaccharide group and at least 60 wt.-% and in particular 80 to 100 wt.-% of the carbohydrates of the carbohydrate component B belong to the fructo-polysaccharide group.

Applicants are also submitting herewith for the Examiner's consideration, two scientific articles i.e. "Dosage-Related Bifidogenic Effects of Galacto- and Fructooligosaccharides in Formula-Fed Term Infants" and "Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants". The results obtained in the article "Supplementation of a bovine milk ..." have been performed with the mixture described in example 1 of the present application. Example 1 of the present application deals with the most important mixture, a combination of GOS (galacto-oligosaccharides) and FOS (fructo-oligosaccharides). See in particular claims 44 and 47. In this regard, these claims specify structure. This structural combination must be suggested by the teachings of the prior art. Applicants specification may not be relied upon to provide this teaching. Hindsight is not

permitted to fill in for the prior art the missing motivation and the expectation of success required to establish a prima facie case of obviousness.

Thus it is clear from the language of claim 1 that carbohydrate components A and B are of **different size**. In addition, claim 14 also requires that carbohydrate component A has a **different structure** than carbohydrate component B. Since the recitation of the different structure is in addition to the above-described difference in size between components A and B, it is clear that components A and B differ in size as well as structure. In other words, in addition to differences attributed to the number of monosaccharide units contained in the components, these components also have additional structural differences.

The above-noted differences in terms of both size and structure are self-evident from the previous versions of claim 14. However, applicant has amended claim 14 to particularly recite this feature which was clearly inherent or at least implicit from the earlier versions of claim 14. Thus claim 14 presently recites that "the carbohydrates/saccharides of carbohydrate component A **have a different structure and a different size** than the carbohydrates/saccharides of carbohydrate component B.

With respect to the aforementioned differences in size and structure, the Examiner's attention is directed toward the paragraph bridging pages 8-9 of the specification wherein it is stated that although components A and B primarily differ in size, mixtures which are particularly efficient also require that component A and component B are of a different structure. Examples of differences in structure are provided in this same paragraph. For example, differences in structure may pertain to the glycosidic bonding. For example, one component may be α-galacto oligosaccharides while the other component may be β-galacto oligosaccharides. It is also noted in the first full paragraph on page 9 of applicant's specification that the aforementioned

structural difference may mean that the two components belong to two different "classes" of carbohydrates.

Applicant submits that the prior art fails to disclose or suggest either of these two limitations. In this regard it is to be noted that column 2, lines 1-6, of Green et al. describes three components of their composition. One of the components is an insoluble non-starch polysaccharide which obviously cannot correspond to applicant's components A or B since components A and B in applicant's invention are soluble. Thus, the remaining two components mentioned in column 2, lines 1-6 (an oligosaccharide component and a non-starch polysaccharide component) must be the components which correspond to applicant's components A and B. However, Green et al. fail to make any provision for assuring that these two components differ both in size and structure. In this regard it is to be noted that Green et al. state that the oligosaccharide component may comprise **any saccharide** containing at least two and up to twenty monosaccharide units, whether a starch (α-glucan) or non-starch type (see column 3, lines 4-7). Thus, it is clear that since Green et al. can use any saccharide for the oligosaccharide component, Green et al. fail to disclose or suggest that this component must differ from the other component in terms of a structural difference in addition to a difference in size.

In view of the above, it is clear that the cited references do not disclose or suggest all of the claimed features of applicant's invention.

Firstly, as discussed above, two carbohydrate components of different chain length have to be present.

Secondly, not only at least 80 weight percent of the carbohydrates/saccharides of carbohydrate component A have to have a prebiotic effect, but also at least 80 weight percent of the carbohydrates/saccharides of carbohydrate component B have such a prebiotic effect.

Since the carbohydrates used as carbohydrate component A and also as carbohydrate component B have to have a prebiotic effect, it should be clear that applicant is not claiming the prebiotic effect of the carbohydrates since the functional requirement is a prerequisite for choosing the appropriate carbohydrates.

Green et al. fail to disclose or suggest the above-noted choice of appropriate carbohydrates. In fact, not all of the saccharides as taught by Green et al. can be used in applicant's invention. In this context applicant directs the Examiner's attention to page 4, last paragraph of the Office Action. There it is pointed out that as far as component B is concerned, Green et al. discloses a composition, which may contain pectin among other ingredients. In this context it must be noticed that pectin generally contains at least 200 monomeric units so that it cannot be considered as carbohydrate component B of applicant's invention since carbohydrate component B of applicant's invention contains a maximum of 100 monosaccharide units. This analysis also applies to the other non-starch polysaccharides mentioned in column 2, lines 47-59 of Green et al. In this context for instance gum arabic can be mentioned.

Furthermore, applicant wishes to point out that Green et al. teaches in column 2, lines 47-59 that inulin may be used. Inulin is a polysaccharide which consists mainly of fructose units. However, the inulin normally used is a mixture of polysaccharides of different chain length (i.e., the number of fructose units varies) and therefore comprises both short chain carbohydrates as well as long chain carbohydrates. Even if one would argue now that some of the carbohydrates belonging to inulin would constitute a carbohydrate component A and the others would constitute a carbohydrate component B, it has to be emphasized that the short chain carbohydrates (carbohydrate component A) and the long chain carbohydrates (carbohydrate component B) **have the same structure** since, as discussed above, inulin is a polysaccharide mainly

consisting of fructose units whereby the polysaccharides only differ in chain length, not both structure and chain length as required in applicant's invention.

In view of the above comments and further amendments to the claims, applicant respectfully requests favorable reconsideration and allowance of all of the claims which are currently pending the application.

Respectfully submitted,

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Dosage-Related Bifidogenic Effects of Galacto- and Fructooligosaccharides in Formula-Fed Term Infants

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ABSTRACT

Background: Human milk oligosaccharides have been shown to stimulate selectively the growth of *Bifidobacteria* and *Lactobacilli* in the intestine. In this study, the bifidogenic effect of an experimental prebiotic oligosaccharide mixture consisting of low-molecular-weight galactooligosaccharides and high-molecular-weight fructooligosaccharides was analyzed in 90 term infants.

Methods: Two test formulas were supplemented with either 0.4 g/dL or with 0.8 g/dL oligosaccharides. In the control formula, maltodextrin was used as placebo. At study day 1 and study day 28, the fecal species, colony forming units (cfu) and pH were measured and stool characteristics, growth, and side effects were recorded.

Results: At study day 1, the median number of *Bifidobacteria* did not differ among the groups (0.4 g/dL group, mean [interquartile range] 8.5 [1.9] cfu/g; 0.8 g/dL group, 7.7 [6.1] cfu/g; and the placebo group, 8.8 [6.1] cfu/g) (figures in square brackets are interquartile range). At the end of the 28-day feeding period, the number of *Bifidobacteria* was significantly increased for both groups receiving supplemented formulas (the 0.4 g/dL group, 9.3 [4.9] cfu/g; the 0.8 g/dL group, 9.7 [0.8] cfu/g) versus the placebo group (7.2 [4.9] cfu/g, $P < 0.001$). This effect was dose dependent (0.4 g/dL versus 0.8 g/dL, $P < 0.01$). The number of *Lactobacilli* also increased significantly in both groups fed the supplemented formulas (versus placebo, $P < 0.001$), but there was no statistically significant difference between the group fed formula with 0.4 g/dL oligosaccharides and the group fed formula with 0.8 g/dL oligosaccharides. The dosage of supplement significantly influenced the change in fecal pH ($P < 0.05$) (placebo, pH 5.5–6.1; 0.4 g/dL formula, pH 5.48–5.44; 0.8 g/dL formula, pH 5.54–5.19). Slight changes in the stool frequency resulted in a significant difference between the placebo group and the group fed the 0.8 g/dL formula at day 28 ($P < 0.01$). Supplementation had a significant dose-dependent influence on stool consistency (0.8 g/dL versus placebo, $P < 0.0001$; 0.8 g/dL versus 0.4 g/dL, $P < 0.01$). Supplementation had no influence on the incidence of side effects (crying, regurgitation, vomiting) or growth.

Conclusions: These data indicate that supplementation of a term infant's formula with a mixture of galacto- and fructooligosaccharides has a dose-dependent stimulating effect on the growth of *Bifidobacteria* and *Lactobacilli* in the intestine and results in softer stool with increasing dosage of supplementation. *JPGN 34:291–295, 2002.* **Key Words:** Galactooligosaccharides—Fructooligosaccharides—*Bifidobacteria*—*Lactobacilli*—Term infants—Dosage. © 2002 Lippincott Williams & Wilkins, Inc.

In utero, the fetus is sterile until the rupture of the fetal membranes. During vaginal delivery, the infant will acquire the initial microflora from the mother. After this initial inoculation of bacteria, the intestinal flora is modulated by several extrinsic factors (1–3). The type of diet is one factor that determines the composition of the intestinal microflora of breast-fed infants, which differs from the microflora of bottle-fed infants (4). In breast-fed infants, the intestinal microflora is dominated by *Bifidobacteria* and *Lactobacilli*, and this microbial pattern produces beneficial effects on intestinal function and on development of the immune system (5,6). Although the mechanisms of these effects are very complex and not fully understood, dietary interventions to establish an intestinal microflora dominated by *Bifidobacteria* and *Lactobacilli* are recommended (7–9).

The effect of human milk on the intestinal flora is caused by its content of selective agents that can stimulate the growth of *Bifidobacteria* and *Lactobacilli*. Oligosaccharides, which are a major component of human milk (10), have been identified as a "bifidogenic" factor of human milk (11,12). Recently, human milk oligosaccharides were shown resistant to enzymatic digestion in the upper gastrointestinal tract (13). Nondigestibility and selective fermentation by potentially beneficial bacteria in the colon are prerequisites for a prebiotic effect of dietary ingredients (7–9).

The composition of neutral human milk oligosaccharides is very complex (10–15), and the relation between

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TABLE 2. *Clinical data of the infants enrolled in the study*

Supplementation group	Placebo	0.4 g/dL Oligosaccharides	0.8 g/dL Oligosaccharides
N (M/F)	33 (17/18)	30 (17/13)	27 (12/15)
Gestational age (wk)	39.6 ± 1.9	39.1 ± 2.1	39.8 ± 1.7
Weight at birth (g)	3,243 ± 427	3,228 ± 452	3,287 ± 390
Length at birth (cm)	49.7 ± 2.2	50.1 ± 1.9	50.3 ± 1.4
Age at study entry (days)	6.3 ± 2.1	6.8 ± 1.9	7.2 ± 2.2
Feeding volume (mg · kg^{-1} · d^{-1})	162 ± 58	163 ± 64	173 ± 55
Weight gain during study period (g/d)	36.8 ± 8.3	35.1 ± 6.7	35.9 ± 6.5
Length gain during study period (cm/wk)	0.87 ± 0.16	0.88 ± 0.23	0.87 ± 0.17

rameters was investigated using nonparametric tests. The Kruskal-Wallis test was used for overall group effect. In case of significance, the Mann-Whitney test was performed for single group comparisons.

For comparison of the frequency of positive cultures for *Bacteroides*, *Clostridium* species, *E. coli*, *Enterobacter*, *Citrobacter*, *Proteus*, *Klebsiella*, and *Candida* the χ^2 test was performed.

All tests were performed on an α-level of 5%. P values greater than 0.05 were considered significant. StatView 5.0 software (SAS Institute Inc., Cary, NC, U.S.A.) was used.

RESULTS

At the first study day, the numbers of fecal *Bifidobacteria* did not differ among the groups, median (interquartile range): placebo, 8.8 (6.1) cfu/g; formula supplemented with 0.4 g/dL oligosaccharides, 8.5 (1.9) cfu/g; formula supplemented with 0.8 g/dL oligosaccharides, 7.7 (6.1) cfu/g). During the study period, the number of fecal *Bifidobacteria* increased in both groups that received the supplemented formulas but remained nearly constant in the placebo group. Therefore, at the end of the 28-day feeding period, the number of *Bifidobacteria* in the stools was significantly higher in both groups fed the supplemented formulas than in the stools of the placebo group, median (interquartile range): placebo, 7.2 (4.9) cfu/g; formula supplemented with 0.4 g/dL oligosaccharides, 9.3 (1.6) cfu/g; formula supplemented with 0.8 g/dL, 9.7 (0.8) cfu/g), but there was also a statistically significant difference between the group fed the 0.4 g/dL formula and the group fed the 0.8 g/dL formula ($P < 0.01$) (Fig. 1).

At the beginning of the study period, the number of *Lactobacilli* in the stools did not differ among the groups: placebo, 3.4 (0.2); 0.4 g/dL formula, 3.3 (0.2); 0.8 g/dL formula, 3.4 (0.2). During the study period, the number increased in both groups that received supplemented formulas, and at study day 2, the number was significantly higher ($P < 0.01$) in both groups fed the supplemented formulas than in the placebo group: median (interquartile range): placebo, 3.4 (1.8) cfu/g; 0.4 g/dL formula, 5.9 (1.5) cfu/g; 0.8 g/dL formula, 5.6 (2.1) cfu/g). There was no statistically significant difference

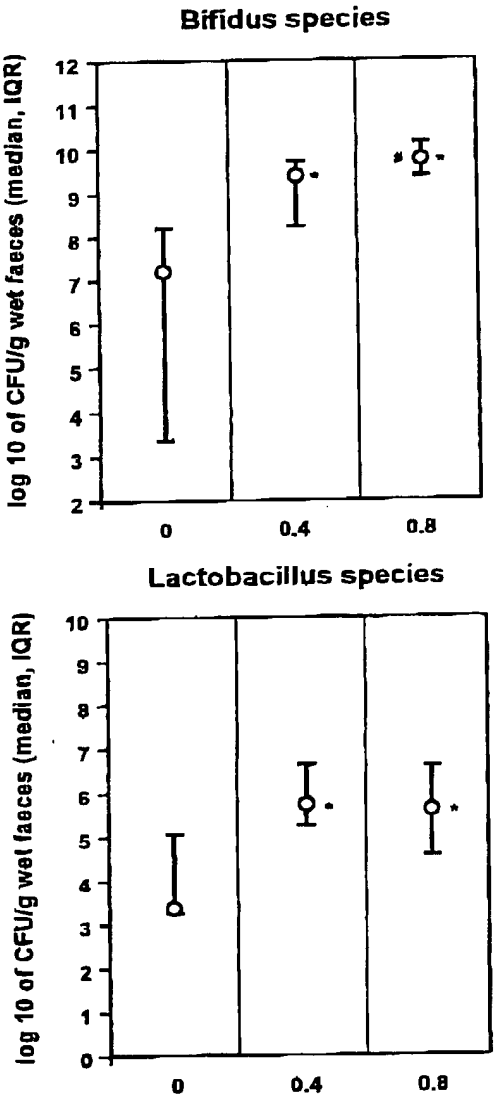

FIG. 1. Influence on the counts of *Bifidobacteria* and *Lactobacilli* of various oligosaccharide supplementations to infant formulas after a 28-day feeding period. IQR = interquartile range.

lar counts of *Bifidobacteria* in all infants after 28 days. This supports the results of a study in adults using different dosages of short-chain fructooligosaccharide (26). In that adult study, a dose-dependent increase of fecal *Bifidobacteria* was also observed.

In our study, galactooligosaccharides derived from lactose were the dominating oligosaccharides in the formula supplement. In human milk, galactose is a major component of human milk oligosaccharides, even if with a different structure. Furthermore, the galactooligosaccharides used in this study have been widely used in infant feeding (17), because they are present in all lactose-reduced or lactose-free products in which lactose has been enzymatically digested (21). To date, no side effects have been reported. More recently, Guesry et al. (27) studied fructooligosaccharides as the only supplement in a formula for term infants at an intake of up to 3 g/day, which is approximately 10 times higher than in our study. They could not demonstrate a bifidogenic effect, nor did they observe side effects. In our study, supplementation also did not influence the incidence of regurgitation, vomiting, or crying, which underlines the safety of the oligosaccharides mixture.

Using the current data, we cannot evaluate to what extent the galactooligosaccharides or the fructooligosaccharides are responsible for the observed effects. However, from the data in the literature (17), a synergistic effect of both ingredients can be assumed. The intensity of the bifidogenic effect of the mixture may indicate that such a synergistic effect took place.

In summary, supplementation of a formula for term infants with a mixture of galacto- and fructooligosaccharides stimulates the growth of *Bifidobacteria* and *Lactobacilli* in the intestine and results in softer stools in a dose-dependent manner. A dosage of 0.4 g/dL results in significant effects, but the effects can be enhanced homogeneously to a level observed in breast-fed infants by increasing the dosage to 0.8 g/dL.

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ORIGINAL ARTICLE

Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants

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Arch Dis Child Fetal Neonatal Ed 2002;86:F178–F181

Background: The establishment of a balanced intestinal microflora which may protect against infection is desirable for the preterm infant.
Objective: To investigate the effect of a preterm formula milk supplement consisting of oligosaccharides in similar proportions to human milk on the faecal flora and stool characteristics of preterm infants.
Study design: To resemble the effect of human milk, an oligosaccharide mixture consisting of 90% galacto-oligosaccharides and 10% fructo-oligosaccharides was used to supplement a standard preterm formula at a concentration of 10 g/l. This supplemented formula was studied in 15 preterm infants, and the results were compared with those found in 15 infants fed a formula supplemented with maltodextrin as placebo. A group fed fortified mother's milk was investigated as a reference group (n = 12). On four days during a 28 day feeding period (1, 7, 14, and 28), the faecal flora was investigated, and stool characteristics, growth, and possible side effects were recorded.
Results: During the study period, the number of bifidobacteria in the group fed the oligosaccharide supplemented formula increased to the upper range of bifidobacteria counts in the reference group. The difference between the supplemented and non-supplemented groups was highly significant ($p = 0.0008$). The stool characteristics were also influenced by the supplement: the stool frequency after 28 days was significantly lower in the control group than in the oligosaccharide supplemented group ($p = 0.0079$) and the reference group ($p < 0.0001$). Over the study period, the stool consistency in the control group became harder, but remained fairly stable in the other two groups. There was no effect of the different diets on the incidence of side effects (crying, regurgitation, vomiting) or on weight gain or length gain.
Conclusion: Supplementing preterm formula with a mixture of galacto- and fructo-oligosaccharides at a concentration of 10 g/l stimulates the growth of bifidobacteria in the intestine and results in stool characteristics similar to those found in preterm infants fed human milk. Therefore prebiotic mixtures such as the one studied may help to improve intestinal tolerance to enteral feeding in preterm infants.

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In the neonatal period, the intestine is colonised in a stepwise process that depends on mode of delivery, environmental factors, bacterial interactions, and the host itself, resulting in colonisation with a complex heterogeneous bacterial flora.[1]

Only a few studies have focused on the early establishment of a balanced intestinal flora in preterm infants,[2 3] and they have shown delayed colonisation with bifidobacteria in preterm infants compared with full term infants. The data also indicate that the influence of prematurity of the intestine of preterm infants is not as important as the influence of extrinsic factors such as type of feeding, antibiotic treatment, and the nosocomial environment of these infants during intensive care.

Preterm infants are particularly vulnerable to intestinal infections, therefore the establishment of a balanced microflora that may protect against infection[4–6] is desirable in such infants.

One extrinsic factor that is important for the timing and quality of intestinal colonisation is feeding. Breast milk seems to favour a more diverse microflora with dominance of bifidobacteria and/or lactobacilli, but the mechanism by which this occurs remains to be clarified.[7] However, the neutral oligosaccharides of human milk[8 9] have been identified as such prebiotic factors. It was recently shown that human milk oligosaccharides are resistant to enzymatic digestion in the upper gastrointestinal tract, which is a prerequisite for a prebiotic effect.[10]

The composition of neutral oligosaccharides in human milk is very complex,[8 9 11–13] and the functional consequences of these different structures are not fully understood.

In this study, we aimed to reproduce the prebiotic effect of human milk oligosaccharides. Because of the high amount of galactose in human milk oligosaccharides, we used available galacto-oligosaccharides derived from lactose as one component of the mixture.[14] The second component was fructo-oligosaccharides extracted from chicory roots with a reduced amount of the low molecular mass fraction (degree of polymerisation (DP) > 10). Fructo-oligosaccharides with DP > 10, as a high molecular mass inulin fraction, have a slower fermentation rate than low molecular mass fructo-oligosaccharides, possibly resulting in fewer side effects such as flatulence.[15]

The mixture is composed in such a way that the size distribution of the molecules is similar to that of human milk oligosaccharides, therefore containing 90% of the low molecular mass galacto-oligosaccharides and 10% of the high molecular mass fructo-oligosaccharides.[15] This combination promotes beneficial intestinal bacteria in a synergistic way so that a maximum number of different species, especially bifidobacteria and lactobacilli, can grow.[16]

Table 1 Composition per 100 ml of the two formulas studied

	Formula without oligosaccharides	Formula with oligosaccharides
Fat (g)	4.4	4.4
Carbohydrates (g)	7.8	7.8
Lactose (g)	6.0	6.0
Maltodextrins (g)	1.8	0.8
Oligosaccharides (g)	0	1.0
Protein (g)	2.4	2.4
Whey/casein ratio	60/40	60/40
Minerals (g)	0.35	0.35
Osmolarity (mOsmol/l)	235–255	235–255
Energy content (kJ)	334	334

The prebiotic effects of both compounds have been shown in several human studies,[9 15 17–19] but to our knowledge they have never been used in combination.

In this study, the effect on the faecal microflora of supplementing a preterm formula with such an oligosaccharide mixture, with particular respect to bifidobacteria, was investigated in preterm infants.

PATIENTS AND METHODS

Preterm infants with a maximum gestational age of 32 weeks according to Dubowitz and Dubowitz[20] admitted to the neonatal intensive care unit of the Hospital Mangiagalli, Milan, were eligible for the study. The study protocol was approved by the ethical committee of the hospital, and informed parental consent was obtained for each infant before enrolment in the study.

For all infants, enteral nutrition was started with pasteurised mother's milk. When a volume of 80 ml/kg/day was tolerated, the milk was supplemented with a commercially available human milk fortifier. When the neonatologist in charge decided to start formula feeding because the mother was no longer able to provide milk, the infants were randomly assigned to one of two formula groups. The compositions of the two formulas were, apart from the supplemented oligosaccharides, identical (table 1). The feeding regimen was performed according to the practice of the hospital and was not influenced by the study protocol. A group fed fortified mother's milk matched for sex and gestational and postnatal age was investigated as the reference group. Table 2 gives the most relevant clinical data of the infants studied.

In the experimental formula, a mixture of fructo-oligosaccharides and galacto-oligosaccharides was added to an otherwise standard preterm formula. As it was the intention to reproduce the spectrum of molecular masses of human milk, the relation between galacto-oligosaccharides and fructo-oligosaccharides was a matter of some experiment. On the basis of our own analytical data,[12] a mixture of 9 parts galacto-oligosaccharides and 1 part fructo-oligosaccharides was found to closely resemble the spectrum of molecular masses of the neutral oligosaccharide fraction of human milk. The concentration of the mixture in the formula was adapted to the concentration of neutral oligosaccharides in human milk—that is, 10 g/l.[12] In the control group, a similar quantity of maltodextrins was added as placebo.

The first day of full formula feeding was defined as measurement day 1. Measurements were repeated after seven, 14, and 28 days. On each of these days, faecal flora was investigated and stool characteristics as well as possible side effects were recorded.

Microbiological analysis of fresh stool sample was performed after suspension, homogenisation, and dilution in physiological saline solution. For analysis of bifidobacteria and lactobacilli, 1 ml of the diluted suspension was inoculated into 10 ml Soybean Casein Digest Agar and incubated at 37°C for five hours. The layer of Soybean Casein Digest Agar was then overlaid with 10 ml Rogosa Agar. After microaerophilic incubation for two days at 37°C, single colonies were counted and identified on the basis of their morphology and biochemical reactions as described previously.[21] The faecal samples were also analysed for *Bacteroides*, *Clostridium* species, *Escherichia coli*, *Enterobacter*, *Citrobacter*, *Proteus*, *Klebsiella*, and *Candida*.

The oligosaccharides were analysed by matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS) as described previously.[12 13 22] For each spectrum, a sum of 50 single spectra was recorded in linear positive ion mode (Voyager DE-STR: Applied Biosystems, Langen, Germany).

Stool characteristics were evaluated on the basis of a questionnaire about frequency and consistency (score 1–5: 1 = watery; 2 = soft; 3 = seedy; 4 = formed; 5 = hard). The consistency of each stool of the respective day was recorded, and the mean of all scores was used to characterise the stool consistency of the respective day.

In addition, the incidence of crying (score 1–3: 1 = seldom; 2 = normal; 3 = often), regurgitation (score 1–3: 1 = none; 2 = once or twice; 3 = more than twice), and vomiting (score 1–3: 1 = none; 2 = once; 3 = more than once) was recorded on the basis of the nurse's report.

For all infants, body weight was measured on each measurement day using a balance with an accuracy of ± 5 g. The crown-heel length was measured at the start and end of each feeding period using a special board for newborn infants with an accuracy of ± 1 mm.

Statistical analysis

All data are given as means (SD). An overall group effect on a measured variable was evaluated by one way analysis of variance. If significant, this was followed by Scheffé post hoc tests for single group comparisons. All tests were performed at an α

Table 2 Clinical characteristics of the study population

	Control formula (without OS)	OS formula	Human milk (reference)
Number (M/F)	15 (8/7)	15 (7/8)	12 (7/5)
Gestational age (weeks)	31.2 (0.6)	31.5 (0.5)	31.4 (0.9)
Weight at birth (g)	1585 (241)	1600 (229)	1601 (321)
Length at birth (cm)	41.4 (2.0)	41.4 (1.9)	41.6 (2.9)
Head circumference at birth (cm)	29.6 (1.8)	29.9 (1.2)	29.9 (1.9)
Age at study entry (days)	8.5 (1.8)	8.3 (3.0)	7.9 (3.2)
Feeding volume (ml/kg/day)	175.6 (19.5)	171.5 (19.8)	186.7 (20.3)
Weight gain during study period (g/day)	29.8 (4.1)	29.8 (4.0)	29.7 (3.3)
Length gain during study period (cm/week)	0.98 (0.05)	0.99 (0.05)	1.01 (0.04)

Values are mean (SD).
OS, Oligosaccharides.

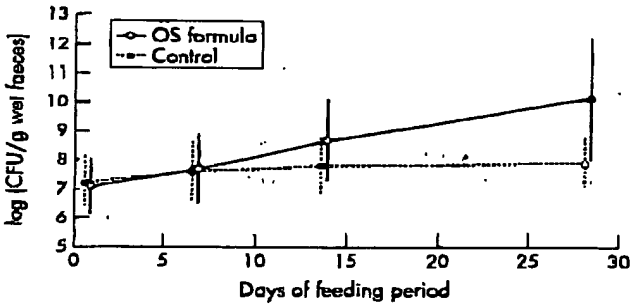

Figure 1 Number of bifidobacteria (mean (SD) log of colony forming units (CFU)/g wet faeces) in the group fed the experimental formula with oligosaccharides (OS formula) and the group fed the unsupplemented control formula (Control). The shaded area indicates the reference range for infants fed human milk (mean (SD) 8.9 (1.8)). The difference between the groups at 28 days was highly significant ($p = 0.0008$; t test).

level of 5%. $p < 0.05$ was considered significant. StatView 5.0 (SAS Institute Inc) was used for the analyses.

RESULTS

The molecular mass distribution of the mixture containing 90% galacto-oligosaccharides and 10% fructo-oligosaccharides as measured by MALDI-MS is similar to that found in the neutral oligosaccharide fraction of human milk (data not shown).

Bifidobacteria were detectable on the first measurement day in all faecal samples. There was no difference between the two formula groups, and both were in the range of the reference group. During the study period, the number of faecal bifidobacteria increased significantly with time in both groups receiving formula ($p < 0.05$), but this increase was only marginal in the group fed the control formula (fig 1). After the 28 day feeding period, the number of bifidobacteria in the group fed the oligosaccharide supplemented formula was in the upper range of the reference group whereas the number in the control group fed the formula supplemented with the placebo was in the lower range. This difference between the two groups receiving formula was highly significant ($p = 0.0008$) (fig 1).

Lactobacilli were also detectable in all infants at the study entry. There was a significant increase in all groups during the course of the study period but there was no significant effect of the diet (data not shown). Neither was there a significant effect of the oligosaccharide supplement on the counts of *Bacteroides, Clostridium* species, *E coli, Enterobacter, Citrobacter, Proteus, Klebsiella,* and *Candida*.

Mean stool frequency increased slightly in the reference group fed mother's milk, remained fairly constant in the group fed the oligosaccharide supplemented formula, and gradually decreased in the placebo group (fig 2). At day 28, the highest frequency was observed in the group fed mother's milk (2.75 (0.8)), which was significantly higher than in the group fed the unsupplemented formula (1.33 (0.6); $p < 0.0001$) and tended to be higher, but not significantly so, than in the group fed the oligosaccharide supplemented formula (2.20 (0.8); $p = 0.1543$).

The stool consistency was also influenced by the diet (fig 2). In the group fed the unsupplemented formula, the score increased during the study period and reached the highest level at the end of the study period. On day 28, the hardest stools were observed in the group fed the unsupplemented formula (score 3.55 (0.8)); they were significantly harder than in the group fed the oligosaccharide supplemented formula (score 2.74 (0.7); $p = 0.0102$) and in the reference group (score 2.33 (0.6); $p = 0.0003$).

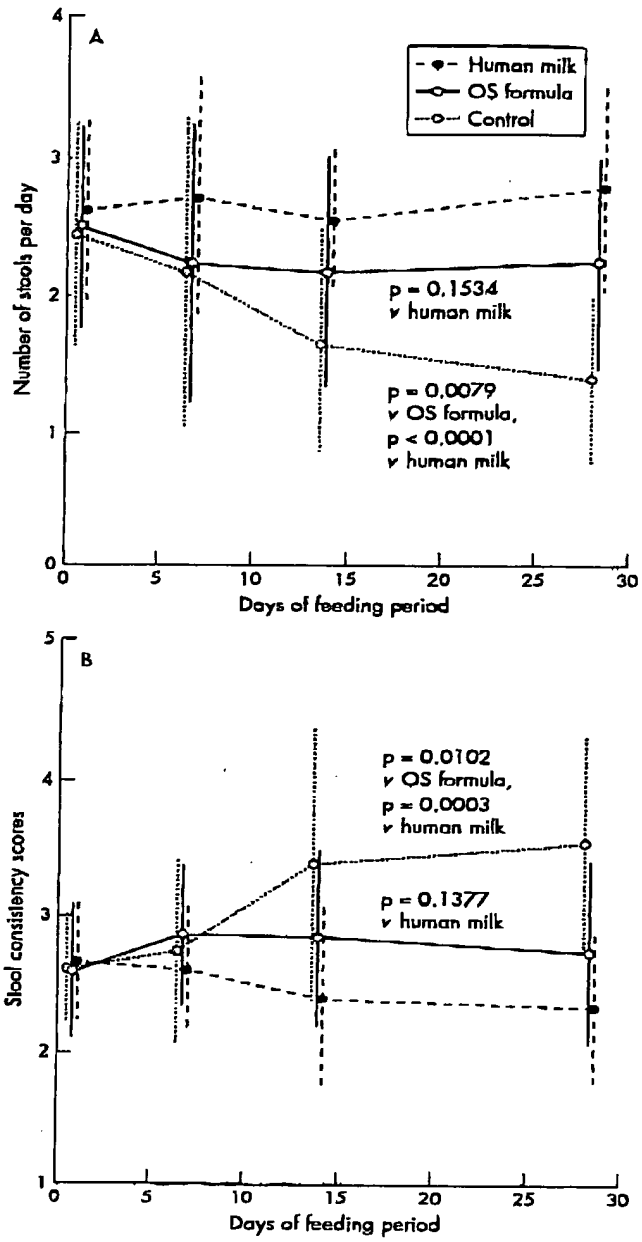

Figure 2 Effect of the formula supplemented with oligosaccharides (OS formula) and the unsupplemented formula (Control) on (A) stool frequency (mean (SD)) and (B) consistency scores (mean (SD); 1 = fluid; 2 = soft; 3 = seedy; 4 = formed; 5 = hard) during the 28 day study period in comparison with the reference group fed human milk. Group differences shown at 28 days were evaluated by analysis of variance followed by Scheffé post hoc tests.

There was no effect of the different diets on the incidence of crying, regurgitation, or vomiting (data not shown). Weight gain and length gain was similar in all the groups (table 2).

DISCUSSION

The prebiotic oligosaccharide mixture used in the study was designed to stimulate growth of bifidobacteria. The data show that supplementation of a standard preterm milk formula with this mixture can significantly stimulate bifidobacterial growth, indicating a bifidogenic effect of the oligosaccharide supplement. In many studies on preterm infants, a delay in the establishment of a bifidogenic flora has been observed. In this study, the first effect of the supplementation on the

number of faecal bifidobacteria was already observed after 14 days, which underlines the bifidogenic potency of the supplement. However, the effect of the supplement on the number of bifidobacteria was more pronounced after 28 days.

A prerequisite for a bifidogenic effect of a dietary ingredient is its resistance to digestion during passage through the small intestine. Even though the digestibility of the supplemented oligosaccharides was not measured in this study, the clear bifidogenic effect indicates that their absorption was as low as found for those in human milk.[10]

The increase in the number of faecal bifidobacteria was accompanied by increased stool frequency and a significant change towards softer stools. Increased frequency and softer stool consistency is an effect of the supplementation that is of practical importance because hard stools and obstipation are common problems that limit the tolerance of preterm infants to enteral feeding.[23] Only 10 g/l of the studied mixture or placebo was added to the formula, which increases the osmolarity (about 5 mOsmol/l) only slightly. Thus, the effect of the supplementation on osmolarity cannot explain the different effects on the stool characteristics, which are probably influenced by the changes in intestinal flora: this is in line with many studies.

A bifidogenic effect of galacto-oligosaccharides[14 19] and fructo-oligosaccharides[15 17 18] has previously been shown. In this study, the strong bifidogenic effect may be explained by a synergistic effect of galacto- and fructo-oligosaccharides—for example, by promoting different strains of bifidobacteria[16]—or, as indicated by in vitro studies, by providing prebiotic substrate to more distal regions of the colon because of the slower rate of fermentation of the high molecular mass inulin fractions.[15] However, as the galacto- and fructo-oligosaccharides were not studied independently, we cannot comment on the individual contributions of the two oligosaccharides to the observed effects.

The study was conducted in healthy preterm infants who tolerated complete enteral nutrition and were not receiving antibiotic treatment—that is, a very selected population. In addition, enteral nutrition in all infants started with mother's milk for several days, which may have influenced the intestinal colonisation at study entry. In fact, bifidobacteria were detected in all infants on the first day of measurement. Therefore the study cannot answer whether such a bifidogenic effect would be observed if intestinal colonisation were severely compromised—for example, after intensive antibiotic treatment.

Studies in adults indicate that dietary fructo-oligosaccharides may lead to side effects, in particular flatulence. In a study using fructo-oligosaccharides during infancy with an intake of up to 3 g/day, however, no such effects were observed.[24] In the present study also, in which only 0.3 g fructo-oligosaccharides were ingested a day, no side effects occurred.

The quantity of oligosaccharide supplement used was 10 g/L to resemble the amount of neutral oligosaccharides found in human milk.[12] The strong and rapid effect on the bifidus flora and stool characteristics suggests that a lower dose may have been sufficient to achieve positive effects on the intestine. This requires further investigation.

In summary, supplementation of a preterm formula with a mixture of galacto- and fructo-oligosaccharides stimulates growth of bifidobacteria and results in stool frequency and consistency similar to those found in preterm infants fed human milk. Thus prebiotic mixtures such as that studied here may help to improve intestinal tolerance to enteral feeding. Further studies on the optimal relation between the different compounds and the dose of the mixture are necessary.

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